SOME HYMENOMYCETES FORMING MYCORRHIZAE WITH PINUS STROBUS L.

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With plates 68 to 71

THE CLASSICAL EXPERIMENTS of Elias Melin (1922) yielded the first conclusive evidence that the Hymenomycetes are involved in the mycorrhizal associations of forest trees. The only fully satisfactory means available for exploring such relationships, namely, the artificial association of tree seedlings with fungi in pure culture, was employed.

In earlier experiments (Melin, 1921) mycorrhizal fungi (*Mycelium radicis* subsp.) were isolated from tree roots and their relationships to tree seedlings demonstrated. By 1925 species among the genera *Lactarius, Russula, Cortinarius, Tricholoma, Amanita,* and *Boletus* had been shown to form mycorrhizaé with *Pinus, Picea, Larix, Betula,* or *Populus* (Melin, 1922, 1923a, 1923b, 1924, 1925a, 1925b, 1925c). Hammarlund (1923) and Masui (1927) have subsequently reported successful syntheses in pure culture.

Precise knowledge on the etiology of the mycorrhizal habit of American Pines is fundamental to studies of their nutrition.[1] With this larger problem in view, a series of syntheses experiments were pursued with *P. Strobus* L. and *P. resinosa* Ait. in association with species of *Boletinus, Boletus, Lactarius, Russula, Amanita,* and *Mycelium radicis* subsp. cultured in both Sweden and America (See Table 1). The study was initiated in Professor Melin's laboratory at the Mycological Laboratory, Royal Academy of Forestry, Stockholm, in the fall of 1929.

Descriptions of technique used in syntheses experiments have been published by Melin (1921, 1923a, 1925a). But Melin has been so frequently misquoted and his technique so often incorrectly followed that recapitulation in connection with the present work is desirable.

Cultures of known fungi were obtained by means of tissue isolations from the pileus and upper portion of the stipes of young sporophores. The following fungi were obtained in culture:[2]

[1]Absorbing roots (short roots, "Saugwurzeln") of Pines in natural habitats are completely mycorrhizal. Thus nutrients entering the tree by these channels must first pass through a fungal mantle that entirely separates the root cells of the tree from the soil.

[2]*Boletinus porosus* and *Boletus castaneus* were identified by Mr. C. L. Krieger, Washington, D. C., *Lactarius chrysorheus* by the junior author and all other specimens by Professors E. Melin and T. Lagerberg.

Lactarius chrysorheus Fr.
" *deliciosus* (L.) Fr., Plate 68, G.
" *subdulcis* (Bull.) Fr.
Russula fragilis (Pers.) Sing.
" *puellaris* Fr.
Amanita muscaria (L.) Fr., Plate 68, D.
Boletus chrysenteron (Bull.) Fr.
" *piperatus* Bull.
" *granulatus* L., Plate 68, B.
" *luteus* (L.) Fr.
" *castaneus* Bull., Plate 68, F.
" *bovinus* (L.) Fr., Plate 68, E.
" *edulis* Bull.
Boletinus porosus (Berk.) Peck, Plate 68, A.

The plan of the work included syntheses experiments with *Mycelium radicis* subsp. isolated from both American and Swedish conifers. The technique developed by Melin (1921, 1925a) for isolating mycorrhizal fungi was employed. Essentially this consists of: (1) selecting comparatively young and clean long-roots bearing mycorrhizae; (2) washing these thoroughly in a strong stream of tap-water; (3) cutting the roots into short lengths, each bearing one mycorrhiza; (4) surface sterilizing the latter in 0.1 per cent bichloride of mercury; and (5) rinsing in three or more changes of sterile water. The time required for surface sterilizing small mycorrhizae of the forked (*Pinus*) or racemose (*Picea*) types was two to five seconds (Melin, 1923a, p. 125). A considerable number of contaminations were inevitable, but longer treatments are lethal to the true endophytes. For tuberous mycorrhizae of Pine one minute usually did not prove injurious. After rinsing, the pieces were placed either in agar petri dishes or on nutrient liquid or agar in test tubes. Uncontaminated pieces were later transferred to suitable culture media.

All of the culture media employed contained, or consisted of, malt extract (See Melin, 1925a, p. 10). For the more sensitive fungi (obtained from either known sporophores or from roots) 5 per cent malt extract, sterilized by passage through a Berkefeld filter, was a suitable medium. Rapidly growing forms (*Boletus bovinus*, etc.) were cultivated on autoclaved 5 per cent malt extract with 2 per cent agar (See Table 1). In the latter case a minimum pressure and period of sterilization was conducive to rapid growth of the organisms.[1]

[1]The American brands of malt extract experimented with were not suitable. They are apparently evaporated at high temperatures, which destroy some nutritive properties. We have used Liebig's malt extract obtained from Apoteksvarucentral Vitrum, Stockholm.

TABLE 1

DATA ON FUNGAL CULTURES

Fungus	Place collected	Date	Most abundant trees in stand	Diam. growth of colonies in mm.	Color of hyphae	Color change of media	Type of Colony	Remarks
Lactarius chrysorheus	Westtown, Pa.	8/26/29	pure P. Strobus plantation	10–12 (ME)[c]	yellowish white	slight	loose, submerged	
Lactarius deliciosus	Djursholm[a]	9/22/29	Pinus, Betula	30–35 (ME)	yellow to whitish yellow	slight	loose, submerged (ME) aërial, submerged (MA) (Pl. 68)	Cultivated on solid media 4 mos. after isolation. Formed strands in culture with Pine.
Lactarius subdulcis	Djursholm	9/20/29	Pinus, Picea	3–4 (ME)	white	slight	compact, submerged	
Russula fragilis	Djursholm	9/20/29	Pinus, Picea	2–4 (ME)	white	slight	compact, submerged	
Russula puellaris	Djursholm	10/15/29	Betula, Pinus	2–4 (ME)	white	slight	compact, submerged	
Amanita muscaria	Djursholm	9/22/29	Betula	5–7 (MA)[f]	white	slight	compact, submerged (ME) compact, aërial (MA) (Pl. 68)	
Boletus chrysenteron	Experimentalfältet[a]	10/3/29	Quercus	2–4 (ME)	light brownish yellow	slight	compact, submerged	
Boletus piperatus	Djursholm	10/28/29	Pinus, Picea	8–10 (ME)	lemon yellow	dark brownish	loose, aërial	
Boletus granulatus	Djursholm	10/9/29	Pinus, Betula	50–55 (MA)	white, brownish with age	light brownish	loose, aërial with strands	Formed strands when associated with seedlings in pure culture.
Boletus luteus	Djursholm	9/20/29	Pinus, Betula	70–75 (MA)	white, brownish with age	dark brownish	aërial (Pl. 68)	
Boletus castaneus	Ansonia, Pa.	8/24/29	Tsuga, Betula	57–62 (MA)	white	pinkish	aërial even (Pl. 68)	

TABLE 1—Continued

DATA ON FUNGAL CULTURES

Fungus	Place collected	Date	Most abundant trees in stand	Diam. growth of colonies in mm.	Color of hyphae	Color change of media	Type of Colony	Remarks
Boletus bovinus	Djursholm	9/20/29	Pinus, Picea	65–70 (MA)	white, brownish with age	dark brownish	aërial (Pl. 68)	
Boletus edulis	Djursholm	9/20/29	Pinus, Betula	3–4 (ME)[c]	white	none	compact, submerged	
Boletinus porosus[d,e]	Warren, Pa.		Betula, Fagus, Tsuga, Prunus	64–69 (MA)[f]	deep brownish yellow	dark brown to black	crusted on surface of media, margin serrate	Numerous clamp connections
M.r. (Pinus) Strobi 1[e]	Ansonia, Pa.	Apr. '29	Conifer, hardwood	33–37 (MA)	white, brown with age	brownish	aërial	
M.r. (P.) sylvestris 1	Tureberg[a]	9/29/29	Pinus, Picea, Betula	35–40 (MA)	white, brown with age	brownish	aërial	
M.r. nigrostrigosum[b]	Kulbäcksliden[a]	4/2/30	Pinus, Betula, Picea		jet black	none	compact, submerged (ME) compact, aërial (MA)	
M.r. atrovirens 1[e]	Keene, N. H.	4/17/29	Pinus	85–95 (MA)	grayish green	none	See Melin 1923	
M.r. atrovirens 2[b]	Kulbäcksliden	4/2/30	Pinus, Betula, Picea	60–65 (MA)	grayish green	none	See Melin 1923	
M.r. (Picea) Abietis 1	Tureberg	9/29/29	Pinus, Picea, Betula	15–18 (ME) 10–12 (MA)	white-pinkish	brownish black	loose, submerged (ME)	Numerous clamp connections

a. Sweden.
b. Isolated from seedlings grown in soil-sand pot experiments by P. R. Gast. Soil from Brända Holmen, Kulbäcksliden Experimental Forest, Vindeln, Sweden.
c. ME—5 per cent liquid malt extract sterilized by passage through Berkefeld filter. MA—5 per cent malt extract, 2 per cent agar sterilized by autoclaving (Liebig's Malt Extract).
d. Isolated by A. H. Hough, Allegheny Forest Experiment Station. Subsequent isolations made by us in 1932 check exactly with original culture.
e. Cultured on an American brand of desiccated malt extract.
f. Those marked "(MA)" represent growth for 30 days on 30 ml. 5 per cent malt agar in 100 mm. Petri dishes.

In designating the imperfect stages of mycelia isolated from roots we have followed Burgeff (1932, p. 147) and included the generic names (in parenthesis) as well as the specific names of the vascular plants from which the mycelia were isolated. These names are of value to the individual investigator and likewise conveniently serve to inform the reader of the identities of the hosts from which they were isolated. In those cases where the mycelial characters are so marked that the fungus in question may easily be recognized by other investigators (particularly when the fungus is associated with more than one vascular plant), the name of the plant from which it was isolated is of less value and may be substituted by a descriptive specific name. Examples of such fungi are *Mycelium radicis atrovirens* Melin, and *M. r. nigrostrigosum* Hatch.

The following fungi were isolated from mycorrhizae of *Pinus Strobus*, *P. sylvestris* L., *Picea Abies* (L.) Karst.: *Mycelium radicis* (*Pinus*) *Strobi 1*, *M. r.* (*Pinus*) *sylvestris 1*, Plate 68, C, *M. r. nigrostrigosum*, *M. r. atrovirens 1*, *M. r. atrovirens 2*, *M. r.* (*Picea*) *Abietis 1*, Plate 68, I.

The tree seeds used in our experiments were obtained through commercial seed houses. They were soaked over-night in water, surface sterilized for two minutes in 0.1 per cent bichloride of mercury, and rinsed in several changes of sterile water. They were then sown on agar in petri dishes, and contaminations and infected seeds were removed with a sterile spatula as they became evident. Early experience demonstrated that the particular sample of *P. Strobus* seeds we used required an after-ripening treatment to obtain even nominal germination. Barton (1928) had shown that cold storage treatment was effective in hastening germination of Southern Pine seeds. We, therefore, surface sterilized seeds of *P. Strobus* and stored them in a frigidaire at four to ten degrees centigrade for a period of two months. Regardless of the acidity of the media (moist filter paper, agar, and peat) which we varied from pH 3.5 to neutrality, germination was uniformly good (approximately 80 per cent).[1] Germination was procured in a constant temperature room at 25 degrees centigrade. As they germinated, the seeds were transferred directly into the culture chambers.

The culture technique developed by Melin (1925a, etc.) was employed in the syntheses. Fluvio-glacial sand was screened and that portion having particle sizes between 0.5 and 2.0 mm. was used (adequate aëration of the substratum is not possible in an undrained flask if smaller particles are included). The sand was boiled in concentrated

[1]Barton (1930) has published a second paper in which success is reported with low temperature treatments of *P. Strobus*.

hydrochloric acid for two hours, washed several hours in running tap-water, and finally in five changes of distilled water. It was dried in an oven, and 150 gram samples were weighed into 300 ml. Erlenmeyer flasks. The nutrient solution added to this substratum contained the following:

KH_2PO_4	0.5 grams
$CaCl_2$	0.05 "
NaCl	0.025 "
$MgSO_4 + 7\ H_2O$	0.15 "
$(NH_4)_2PO_4$	0.25 "
Iron citrate	0.025 "
Dextrose	0.5 "
Distilled water	1. litre

Thirty-seven ml. of this solution were added to each flask. The pH of the solution was 6.57. After autoclaving with the sand, this changed to approximately 4.2.

The nutrient conditions in our experiments differed from those in Melin's (1923a, p. 159). We used a less concentrated solution and likewise added 37 rather than 50 ml. of solution to each flask. Further, the growth of *P. Strobus* is considerably greater in culture than that of *P. sylvestris* seedlings. The differences in the nutritional conditions of the two sets of Pines were, therefore, quite marked. Since $\frac{\text{root}}{\text{shoot}}$ growth is greater when nutrients are present in comparatively small quantities, it was argued that more short roots would develop. The probability of obtaining mycorrhizae would therefore be enhanced. It is not necessary, however, to obtain large numbers of mycorrhizae in syntheses experiments, as has been emphasized by McArdle (1932, p. 314). The unquestionable demonstration of the presence of only one typical mycorrhiza is adequate proof that the organisms concerned enter into mycorrhizal association with each other.

The assembled culture chamber consisted of the Erlenmeyer flask with an inverted beaker over the cotton plug (Plate 70, D). Between March 23, 1930 and April 9, 54 of these units, each with a germinated seed of *P. Strobus*, and 52 units with *P. resinosa* seedlings, were set up. They were placed in the diffused light of a west window and received direct solar radiation, passing through the foliage of a large oak tree, late in the evening only (Plate 70, D). On June 5th to 7th the seedlings were inoculated with all of the fungi listed above. The sub-cultures from which inoculations were made were less than ten days old. A number of seedlings were reinoculated August 21.

Half of the flasks were opened during November 1930. These were

chiefly *P. Strobus* syntheses.[1] The remainder were kindly cared for by Professor Melin until the fall of 1931, when the seedlings were placed in fixing solution and shipped to the authors in America. Except for the latter, the substrata in all flasks were tested for contaminations at the close of the experiment by placing sand from the flasks on malt agar media in culture tubes. Of thirty-five cultures of *P. Strobus* opened in November 1930, three were contaminated. A number of contaminations from seed-coat infections with *P. resinosa* were observed during the course of the experiment. Similar contaminations with *P. Strobus* did not occur, since these seeds were on moist agar for nearly three months before they were transferred to the flasks and, consequently, seed-coat infections were eliminated.

Fixation was with Karpchinko solution; it did not prove particularly good for mycorrhizal details. The roots were embedded in paraffin for sectioning; both the butyl and ethyl alcohol series were used. Gross photographs of mycorrhizal roots were made in distilled water between glass plates. Microplanar or Tessar lenses, and Ilford panchromatic soft gradation plates with a Wratten B (red) filter were used. The staining technique employed will be reported by Dr. K. D. Doak in a future communication.

RESULTS

Typical ectotrophic mycorrhizae were form with *P. Strobus* by twelve of the fungi investigated:

Lactarius chrysorheus Fr.,	Plate 70, E
" *deliciosus* (L.) Fr.,	" 69, A; Plate 71, A
Amanita muscaria (L.) Fr.,	" 69, C; " 71, D
Boletus castaneus Bull.,	" 70, I
" *bovinus* (L.) Fr.,	" 70, F
" *luteus* (L.) Fr.,	" 69, B
" *granulatus* L.,	" 70, H
Boletinus porosus (Berk.) Peck,	" 70, G; Plate 71, C
Mycelium radicis nigrostrigosum	
" " (*Picea*) *Abietis* 1	Plate 71, B
" " (*Pinus*) *Strobi* 1	
" " (*Pinus*) *sylvestris* 1	

The mycelia of the *M. r. atrovirens* type, as in Melin's experiments, overgrew the aërial parts of the seedlings and failed to exhibit any indications of mycorrhiza-formation (Plate 70, A). The mycelia

[1] *P. resinosa* grew very poorly in the medium used in these experiments. Root development was adequate for mycorrhiza-formation in only two or three cases. These will be reported elsewhere.

of all other fungi failed to develop in the substrata, and information on their ability to form mycorrhizae was not obtained.

DISCUSSION

Positive results with *Boletinus porosus* adds *Boletinus* to the genera of fungi that have been proved to contain mycorrhiza-forming species. *Lactarius chrysorheus* and *Boletus castaneus* are also added to the list of known mycorrhizal organisms. The remaining fungi (with the exception of those isolated from tree roots) have previously been tested in pure culture with success as follows:

Lactarius deliciosus, with *Pinus mugo* Turra (*P. montana* Mill.), *P. sylvestris* and *Picea Abies* (Melin, 1924, 1925a).

Amanita muscaria, with *Betula pendula* Roth, *B. alba* Roth, *Larix decidua* Mill. (*L. europaea* DC.), *Pinus sylvestris,* and *Picea Abies* (Melin, 1923a, 1925a).

Boletus granulatus, with *P. sylvestris* and *P. mugo* (Melin, 1923a, 1924b).

Boletus bovinus, with *Pinus densiflora* Sieb. & Zucc. (Masui, 1927).

Boletus luteus, with *P. sylvestris, P. mugo, Larix decidua* Mill., *L. occidentalis* Nutt. and *Picea Abies* (Melin, 1923a, 1923b, 1925a).[1]

Concerning *Amanita muscaria,* Melin (1925a, p. 100) reports: "Der Pilz scheint aber den Pflanzen gegenüber eine ziemlich hohe Virulenz gehabt zu haben." A similar tendency was exhibited by our culture and by *Lactarius chrysorheus* in a 6-months synthesis as exemplified by the development of a heavy intercellular net. A culture plant removed after 15 months' association with *A. muscaria,* on the other hand, showed normal infection (Plate 69, C).

Concerning *Boletus bovinus,* Masui (1927) inoculated seedlings of *Pinus densiflora* growing in large test tubes on nutrient agar and reports that the seedlings were killed by this fungus. In Erlenmeyer flasks the fungus grew up the stem of the seedlings (l. c., p. 203 and Plate XI., Fig. 7). We note that in the latter photograph the plugs of the flasks

[1]Masui (1927) conducted syntheses experiments between *Pinus Thunbergii* Parl., *Quercus myrsinaefolia* Bl., *Q. phillyraeoides* Gray, *Q. glauca* Thunb., *Q. mongolica* Fisch. var. *grosseserrata* Rehd. & Wils., *Q. paucidentata* Franch., and a fungus which he isolated from the tuberous (compound) mycorrhizae of the last named Oak. He believed these mycorrhizae were formed by the mycelium of a *Boletus,* the sporophore of which was attached to the mycorrhiza from which isolations were made. He listed this sporophore as *Boletus luteus* (?). In a footnote (1927, p. 195) Masui mentions that Dr. Krieger believes the specimen may be *B. granulatus.* The identity of the culture is, therefore, not certain, and we have excluded these results from the list above, which represents authentic pure culture syntheses only.

were covered. The explanation of the behavior of *B. bovinus* as reported by Masui is, therefore, probably attributable to excessively high humidities. Our culture of *B. bovinus* grew very rapidly, and quickly covered the substrata within the flasks (the only fungus that covered the sand with surface growth), but exhibited no tendency to overgrow the seedlings (Plate 70, C). The mycorrhizae formed by *B. bovinus* possessed hyphal mantles one-fourth of their total thickness.

It is of interest to record here that in some of the cultures two easily separable fungi, *Mycelium radicis nigrostrigosum* and *M. r.* (*Picea*) *Abietis 1*, were associated with the same seedling. Typical mycorrhizae were formed by each of the fungi with short-roots, and in addition *M. r. nigrostrigosum* formed a secondary mantle over several of the mycorrhizae of the *M. r.* (*Picea*) *Abietis* type (Plate 71, B).

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EXPLANATION OF PLATES

Plate 68. Colonies of mycorrhiza-forming fungi grown in 100 mm. Petri dishes on 30 ml. of 5 per cent malt extract (Liebig's) agar for 30 days (60 days for D and 20 days for F) at 25 degrees centigrade, × 1.
A. *Boletinus porosus.*
B. *Boletus granulatus.*
C. *Mycelium radicis (Pinus) sylvestris 1.*
D. *Amanita muscaria.*
E. *Boletus bovinus.*
F. *Boletus castaneus.*
G. *Lactarius deliciosus.*
H. *Mycelium radicis (Picea) Abietis 1.*

Plate 69. Mycorrhizae on roots of *Pinus Strobus* seedlings grown in pure culture.
A. Whole root system of seedling inoculated with *Lactarius deliciosus.* All short roots mycorrhizal, × 4. (Photo, U. S. Dept. Agric.)
B. Mycorrhizae formed by *Boletus luteus,* × 9.
C. Mycorrhizae formed by *Amanita muscaria,* × 9.

Plate 70. A. Seedling of *Pinus Strobus* inoculated with *Mycelium radicis atrovirens 2,* showing mycelial growth on stem and lower leaves, × 2/3. (Top of flask removed for photo.)
B. Uninfected short roots of *P. Strobus.* Seventeen months old seedling, × 4.
C. Seedling of *P. resinosa* inoculated with *Boletus bovinus,* showing mycelial growth over sand substratum, × 2/3. (Top cut from flask for photo.)
D. Cultures of *P. Strobus* and *P. resinosa* in west window of the Royal Academy of Forestry, Stockholm.
E. Mycorrhizae of *P. Strobus* formed by *Lactarius chrysorheus,* × 6. (Photo, U. S. Dept. Agri.)
F. Mycorrhizal short roots of *P. Strobus* formed with *Boletus bovinus,* × 9.
G. Mycorrhizae of *P. Strobus* formed with *Bolctinus porosus,* × 8.

H. Mycorrhizal roots of *P. Strobus* formed with *Boletus granulatus*, × 6.
I. Mycorrhiza of *P. Strobus* formed with *Boletus castaneus*, × 10.

Plate 71. Photomicrographs of mycorrhizae of *P. Strobus* from syntheses experiments, showing distribution of mycelia between the cortical cells, and mantle structure.
A. Inoculum—*Lactarius deliciosus*, medial, longitudinal section, × 400. (Photomicrograph by K. D. Doak.)
B. Inocula—*Mycelium radicis* (*Picea*) *Abietis 1* and *M. r. nigrostrigosum*, somewhat oblique, longitudinal section, × 400. *M. r. nigrostrigosum* forming a secondary mantle over that of *M. r.* (*P.*) *Abietis 1*. (Photomicrograph by K. D. Doak.)
C. Inoculum—*Boletinus porosus*, medial, longitudinal section, × 370.
D. Inoculum—*Amanita muscaria*, oblique, transverse section, × 460. From 17-months old seedling.

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